to at least 16 days in Amies transport medium containing charcoal. These data will help laboratories adopt safe and reliable procedures to ensure that pneumococcal culturability is not lost during transportation.

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Polymerase chain reaction amplification: effect of dyes and other staining agents employed in clinical microbiology laboratories

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Correct and reliable isolation and identification of bacterial organisms in clinical microbiology is an important function of such service laboratories. Causal organisms of infection may be presented for identification through their growth from enrichment on non-selective/selective media and directly from clinical specimens. To date, such identification has largely relied on phenotypic schema, including initial examination of colonial morphology and Gram stain, which is usually followed by some form of semi-automated identification scheme, generally based on biochemical differentials, such as the API identification schemes or the BBL Crystal scheme. Molecular methodologies, particularly employment of the 16S rDNA polymerase chain reaction (PCR) and sequencing techniques, offer an alternative laboratory mechanism for the identification of such organisms. These have a specific operational advantage when working with non-culturable organisms such as the obligate intracellular organisms *Coxiella burnetii*, *Tophereyma whipplei* or difficult-to-culture organisms such as the slow-growing *Mycobacterium* spp.

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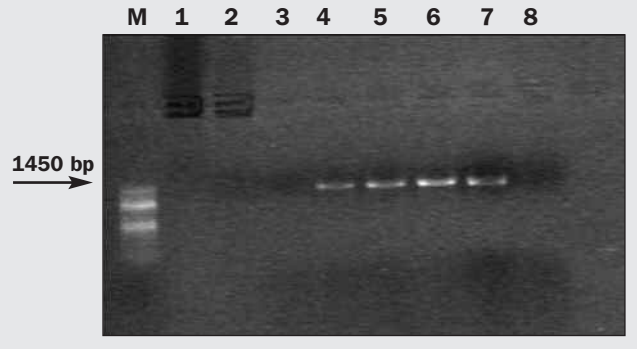

Fig. 1. Effect of the presence of crystal violet on PCR amplification of the 16S rRNA gene in *E. coli* 0157:H7 NCTC12900. Lane M: molecular weight marker (100 bp; Gibco Life Technologies, Paisley, Scotland), lane 1: crystal violet (91.9 μg), lane 2: 10^{-1} dilution (9.19 μg), lane 3: 10^{-2} dilution (0.919 μg), lane 4: 10^{-3} dilution (91.9 ng), lane 5: 10^{-4} dilution (9.19 ng), lane 6: 10^{-5} dilution (0.919 ng), lane 7: positive control (bacterial DNA present/crystal violet absent), lane 8: negative control (PCR grade water; crystal violet and bacterial genomic DNA absent).

Often, such causal organisms are seen in histopathological specimens or bacteriological glass microscope slides, but cannot be confirmed by conventional bacteriological culture techniques. Visualisation of cellular morphologies by light microscopy generally involves the employment of differential stains and dyes, which either serve to aid visualisation (eg staining spores with malachite green) or characterisation of such organisms (eg Gram stain). A wide range of dyes and stains are available and are employed for these purposes; however, there has been relatively little work performed to determine if they have any potential influence on PCR amplification. Previously, in cytopathology, cellular digests from Papanicolaou-stained cervical smears did not yield products from PCR, whereas cellular digests from unstained cervical smears always yielded PCR products.[1] Analysis of individual Papanicolaou stain reagents identified inhibition of PCR by haematoxylin and by aluminium sulphate. These inhibitors could be removed from Papanicolaou-stained cervical smears by destaining the slides with 1% HCl.[1]

The application of molecular techniques for identification and downstream molecular assays, including genotyping or other molecular characterisation assays, on archived material mounted on glass slides has the potential to provide detailed identification and epidemiological information on the causal organism seen initially by light microscopy.

Previously, the authors developed a robust method of removal of fixed oocysts of *Cryptosporidium parvum* from archived material using laser-capture microscopy (LCM).[2]

Table 1. Dyes and stains used and their concentrations, as well as their ability to allow a 16S rRNA gene PCR reaction to amplify successfully.

		Ability to amplify with PCR (serial dilution)					
Dye/stain	Amount per PCR reaction	Neat	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Phenol red (0.1% solution)	18.4 µg	–	+	+	+	+	+
Malachite green (2% solution)	367.5 µg	–	–	–	+	+	+
Brilliant green (0.5% solution)	91.9 µg	–	–	+	+	+	+
Bromocresol purple (0.15% solution)	27.6 µg	–	–	+	+	+	+
Crystal violet (0.5% solution)	91.9 µg	–	–	–	+	+	+
Neutral red (0.1% solution)	18.4 µg	–	–	–	w	+	+
Carbol fuchsin (0.1% solution)	18.4 µg	–	w	+	+	+	+
Lugol's solution (iodine)	183.8 µg	–	–	–	–	+	+

–: no amplification, +: amplification, w: weak PCR reaction.

However, they now intend to examine further the potential problems associated with the removal of bacteriological material from glass slide-mounted material treated with a range of common dyes and stains. Therefore, this study aims to examine the effect of dyes and stains commonly used in clinical microbiology laboratories on PCR amplification and to propose a pragmatic method to overcome any PCR inhibition problems due to such dyes and stains.

A non-toxigenic strain of *Escherichia coli* O157:H7 (NCTC 12900) was employed in this study. The isolate was purified on Colombia agar base supplemented with defibrinated horse blood 5% (v/v) (Oxoid) and was incubated at 37°C for 24 h. Genomic DNA was isolated using the Roche High Purity PCR Template Preparation Kit (Roche, England), following the manufacturer's instructions. All DNA isolation procedures were carried out in accordance with the DNA contamination management guidelines of Millar *et al.*[3] in a class II biological safety cabinet (MicroFlow, England). Extracted DNA was transferred to a clean tube and stored at –80°C prior to PCR amplification.

Eight common dyes and stains used in clinical microbiology laboratories were prepared, as detailed in Table 1. All reaction mixes were set up in a PCR hood in a room separate from that used to extract DNA, and from the amplification and post-PCR room in order to minimise contamination.[3] Dyes/staining agents were incorporated into the PCR reactions at various concentrations (neat – 10^{-5} dilution; Table 1), where the 'neat' concentration represented the normal laboratory working concentration. The PCR reaction mixes (25 µL) contained 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 200 µmol (each) dATP, dCTP, dGTP and dTTP, 1.25 units *Thermus aquaticus* (*Taq*) DNA polymerase (Amplitaq; Perkin Elmer), 0.2 µmol (each) universal 16S rRNA primers 27F (AGT GTT TGA TCM TGG CTC AG) and 1492R (ACG GYT ACC TTG TTA CGA CTT), as previously described,[4] together with 4 µL DNA template containing approximately 50 ng DNA/mL extract, as well as the dyes/stains detailed in Table 1. Following a 'hot start', the reaction mixtures were subjected to the following thermal cycling parameters in a Perkin Elmer 2400 thermocycler: 96°C for 3 min followed by 40 cycles of 95°C for 1 min, 58°C for 30 sec, 72°C for 1 min 30s, followed by a final extension at 72°C for 7 min. During each run, molecular-grade water (Biowhittaker Inc. Maryland, USA) replaced DNA randomly as a negative control, while *Staphylococcus aureus* DNA was included as a positive control.

Following amplification, aliquots (15 µL) were removed and electrophoresed (80 V, 45 min) on 2% (w/v) agarose gel (Gibco, Paisley, Scotland, UK) in TAE buffer (40 mmol/L Tris, 20 mmol/L acetic acid, 1 mmol/L EDTA [pH 8.3]) and stained with ethidium bromide (5 µg/100 mL). Gels were visualised under ultraviolet (UV) illumination using a gel image analysis system (UVP Products, England) and all images were archived as digital (*.bmp) graphic files.

The ability to amplify the 16S rRNA gene in *E. coli* DNA in the presence of individual dyes and stains is shown in Table 1. No PCR amplification was achieved when the undiluted dye/stain solutions were used. The most inhibitory compound was Lugol's solution, which contains iodine and required a 10,000-fold dilution to allow PCR amplification to take place. This was followed by crystal violet solution, which required a 1000-fold dilution to permit PCR amplification. The least inhibitory dye solution was phenol red, which only required a ten-fold dilution to allow PCR amplification. Figure 1 shows PCR amplification of the 16S rRNA gene in *E. coli* O157:H7 NCTC12900 using a serial dilution of a 0.5% (w/v) solution of crystal violet.

Inhibition is an important limiting factor in the application of PCR to diagnostic biomedical science, and has been discussed at length.[5] Many clinical biological substances may act as potent PCR inhibitors and these include blood, bile, faeces and sputa.[5] To date, very little work has been directed towards the examination of potential PCR inhibition due to dyes and stains used in clinical microbiology. In the present study, the authors were able to overcome the problem of PCR inhibition by the serial dilution of dyes/stains, depending on the dye, from 10-fold to 10,000-fold.

In conclusion, this small study demonstrates that several commonly used dyes and stains inhibit PCR amplification. The presence of such compounds in bacteriological material mounted on glass slides may lead to amplification problems when used to identify the presence of causal bacterial agents of infection, and could lead to a false-negative result. Such inhibition may be overcome by the serial dilution of stained material in the range 10-fold to 10,000-fold. Therefore, clinical microbiology laboratories should be aware that such dyes and stains may inhibit PCR amplification and should incorporate appropriate positive and negative controls into their assay protocols to avoid the reporting of false-negative

results. Serial dilution of stained material (to 10,000-fold) may allow such material to be amplified, and this dilution stage should be incorporated into molecular diagnostic standard operating protocols.

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Molecular detection of *Haemophilus influenzae* in COPD sputum is superior to conventional culturing methods

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Chronic obstructive pulmonary disease (COPD) is predominantly a disease of smokers and ex-smokers that not only causes significant morbidity and mortality but also has a strong impact on the quality of life of affected individuals.[1] The disease is characterised by airway inflammation and varying degrees of airflow limitation resulting in irreversible lung damage. Symptoms include breathlessness, coughing, wheezing and sputum production. Disease progression is characterised by increasing disability due to increasing breathing problems, hospital admission and premature death.[2] Unlike the declining mortality associated with other diseases (e.g., cardiovascular disease and stroke), the mortality rate in COPD is actually increasing.[3]

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In COPD the lower airways may be persistently colonised by microbial pathogens (isolated from sputum and bronchial lavage [BAL] samples), even during stable disease. The organisms most commonly isolated are *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*,[4,5] and all are associated with increased inflammation and lung damage.[6] Of these, *H. influenzae* is the most frequently identified[7] and the most common cause of COPD exacerbations.[8,9] In a recent study, it was demonstrated that *H. influenzae* bronchial colonisation in COPD patients was associated with sputum neutrophilia, an identifiable exaggerated inflammatory response, and a significant decline in lung function. In contrast, colonisation with *H. parainfluenza* was not associated with an inflammatory response, suggesting that this organism may not worsen disease severity,[6] even though colonisation has been reported in 20% of COPD patients.[9]

Despite frequent carriage of *H. influenzae*, long-term use of prophylactic antibiotics is not currently recommended as a preventative treatment option[1,10] even though studies demonstrate an associated small decrease in days of illness if used.[11] Concerns regarding the impact of long-term antibiotic use on patient health and the development of antibiotic resistance are thought to outweigh any small benefit. Therefore detection of *H. influenzae* in COPD sputum samples using conventional culturing continues to guide antibiotic usage, and rapid and sensitive molecular detection would allow a prompt clinical response that would greatly improve the quality of patient life and may slow progression of the disease.

Twenty COPD patients were recruited from the Launceston General Hospital with a diagnosis of COPD, respiratory symptoms (e.g., breathlessness, cough, sputum), a smoking history of ≥10 pack years (where one pack year is 20/day for one year or the equivalent number of cigarettes), an FEV1:FVC ratio after bronchodilation of <0.7, and one to two hospital admissions for COPD in the last year. Once enrolled in the study, patients provided a sputum sample (either spontaneously or using a standard saline inhalation procedure[12]) and another sample six months later or upon admission for an acute exacerbation. All patients enrolled in the study gave written informed consent. The study protocol was approved by the Tasmania Human Research Ethics Committee (H0009201).

A total of 36 sputum samples were collected and immediately stored at 4°C before being weighed and then homogenised at 37°C with four volumes of 10% Sputolysin (Calbiochem) for 30 min and plated on chocolate agar supplemented with bacitracin and incubated at 37°C anaerobically for 48 h. Isolates consistent with *H. influenzae* morphology (1–2 mm diameter, flattened convex with or without sunken centres, smooth, entire edge, translucent, greyish) were spread over tryptone soy agar plates with disks of X factor (hemin), V factor (NAD or NADP) and X+V factors, and plates were then incubated as described above. Isolates with growth around the X+V factor only were designated *H. influenzae* and stored. Genomic DNA for the polymerase chain reaction (PCR) assay was extracted from the remaining sputum samples using the method of Reischl *et al*.[13]